In Vitro Cell. Dev. Biol.—Animal 37:343–347, June 2001
© 2001 Society for In Vitro Biology
1071-2690/01 $10.00+0.00

# COCKROACH MIDGUT PEPTIDES THAT REGULATE CELL PROLIFERATION, DIFFERENTIATION, AND DEATH IN VITRO[1]

M. TAKEDA,[2] T. SAKAI, Y. FUJISAWA, M. NARITA, K. IWABUCHI, AND M. J. LOEB

*Graduate School of Science and Technology, Kobe University, Kobe 657-8501, Japan (M. T., T. S.), Suntory Institute for Bioorganic Research, Osaka 618-8503, Japan (Y. F.), Tokyo University of A&T, Fuchu 183-8509, Japan (M. N., K. I.), and Insect Biocontrol Laboratory, USDA, Beltsville, Maryland (M. J. L.)*

## SUMMARY

The number of insect midgut cells is maintained homeostatically in vivo and in vitro. However, during starvation, the midgut shrinks and the rate of cell replacement appears to be suppressed. When they undergo metamorphosis, the internal organs of insects are drastically remodeled by cell proliferation, differentiation, and apoptotic processes, and the net number of cells usually increases. An extract of 1650 midguts of *Periplaneta americana* was fractionated by high-performance liquid chromatography (HPLC) to obtain the peptides that regulate these processes. The HPLC fractions were tested for myotropic activity in the foregut and for effects on cell proliferation or loss in primary cultures of larval *Heliothis virescens* midgut cells and in a cell line derived from the last-instar larval fat body of *Mamestra brassicae.* Some fractions stimulated midgut stem cell proliferation and differentiation, while others caused loss of differentiated columnar and goblet cells. Other fractions stimulated cell proliferation in the larval fat body cells.

*Key words: Periplaneta americana; Heliothis virescens; Mamestra brassicae;* peptide growth factor; proliferation; differentiation; cell death.

## INTRODUCTION

The midgut of insects regulates various metabolic processes and physiological functions such as digestion of foodstuffs (Teo and Woodring, 1989; Valaitis and Boweres, 1993; Valaitis, 1995), absorption of nutrients and electrolytes (Taylor, 1985; Dow and Harvey, 1988), secretion of waste materials, fluid transport, synthesis of hemolymph proteins (Palli and Locke, 1987), nourishment of hemocytes, conversion of ecdysone to 20-hydroxyecdysone, conjugation of ecdysteroids (Weirich et al., 1986; Weirich, 1997), and detoxification (Neal and Rueveni, 1992). The midgut is commonly the attack site for invading microorganisms since its epithelium is devoid of cuticular lining, unlike the foregut and the hindgut. Immunohistochemical studies suggest that the midgut is a rich source of biologically active compounds (Endo et al., 1982; Nishiitsutsuji-Uwo et al., 1985, 1986; Verhaert et al., 1986; Sehnal and Zitnan, 1990; Veenstra et al.,1995), as well as a promising future target for pest control.

Insects undergo starvation from time to time. Upon a shut-off in nutrient inflow, they must shift metabolic gears to convert developmental and physiological programs from an active to a protective mode. We observed a dramatic shrinkage in the midgut caeca in the starved cockroach. Our preliminary data suggest that one of the factors that controls changes in the cockroach midgut nidi, i.e., the nest of new, putative stem cells (Nishiitsutsuji-Uwo et al, 1986), may be serotonin and its metabolites (Takeda et al., 1995). Long ago, Day and Powning (1949) observed that a blood-borne factor stimulated cell proliferation in *Tenebrio molitor* midgut, and named this factor 'midgut regeneration stimulation factor' (MRSF). MRSF could be serotonin, since we observed immunohistochemical reactivity against serotonin in the nidi of the cockroach epithelia and the level of serotonin declined after starvation. Injection of serotonin stimulated the uptake of bromodeoxyuridine in the nuclei of the cells in the nidi (unpublished data). However, it is also possible that the injected serotonin could cause body tissues to trigger the release of 'real' growth factors downstream. From these observations, we concluded that some growth factors either positively or negatively regulate the homeostasis of midgut cell numbers and the constitution of cell types.

Growth factors in invertebrate in vitro culture have been reviewed by Ferkovich and Oberlander (1991). The fully sequenced insect growth factors reported so far are few. The known insect-derived growth factors include the 52-kDa factor isolated from the conditioned medium of the NIH-Sape-4 embryonic cell line of *Sarcophaga perigrina,* a family of imaginal disk growth factors (Homma et al., 1996), polypeptides produced in fat body and embryonic yolk cells that stimulate proliferation in the imaginal disks of *Drosophila melanogaster* (Kawamura et al., 1999), and a family of factors inducing hemocyte spreading, inhibition of embryonic growth, paralysis, and cardiac myoactivity (Strand et al., 2000). The only known peptide growth factors that affect the differentiation of midgut stem cells are two small peptides, fragments of fetuin, isolated from the conditioned medium in which *Manduca sexta* midgut cells were

[1] Mention of products in this article does not imply endorsement by the U.S. Department of Agriculture.

[2] To whom correspondence should be addressed at E-mail: mtakeda@kobe-u.ac.jp

cultured (Loeb et al., 1999). They are the midgut differentiating factors (MDF 1 and 2).

Three peptides with myomodulatory activity have been isolated from the midguts of the American cockroach, *Periplaneta americana* (Sakai et al., in prep.). In this work, we describe the peptides from normally fed cockroach midgut that directly regulate cell proliferation, differentiation, and death. This is the same source that we used for the isolation of myomodulatory peptides. We used in vitro culture systems of two lepidopteran species, *Heliothis virescens* (midgut cell primary culture) and *Mamestra brassicae* (fat body cell line), to bioassay for gut-active peptides (Loeb and Hakim, 1996, 1999), since there was no available cell line with which to study the cockroach midgut system. The *Heliothis* system allowed us to measure peptide activity on cell proliferation, differentiation, and cell death in stem cells and the two types of differentiated midgut epithelial cell, columnar and goblet cells, characteristic of lepidopteran larvae. The activity of peptides on proliferation was measured by counting the total number of cells, while the activities on differentiation and death were measured by counting the number of both types of differentiated cell before and after the addition of peptide fractions to the medium. Fat body cells undergo drastic metamorphic changes, which allowed us to study the activities of peptides on both proliferation and apoptosis.

## Materials and Methods

*Sample preparation.* Midguts from 1650 cockroaches, weighing 34.3 g, were boiled in five times their volume in water for 10 min. After cooling, 4% acetic acid was added to the sample. The material was homogenized on ice, and then centrifuged at 15,000 × *g* for 20 min at 40° C. The supernatant (120 ml) was forced through Mega Bond Elut C18 cartridges (Varian, CA). After washing each cartridge with 0.1% trifluoracetic acid (TFA), the retained material was eluted with 60% acetonitrile in 0.1% TFA, then pooled and concentrated in a rotary evaporator (CI; Hitachi, Tokyo).

*High-performance liquid chromatography purification and peptide sequencing.* The concentrated material was filtered through a 0.45-μm filter (Chromatodisk 4N; Kurabo, Tokyo), and then applied to a C18 high-performance liquid chromatography (HPLC) column (CAPCELLPAK; Shiseido, Tokyo), and eluted with a 70-min linear gradient of 0–70% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min at 40° C. Column effluent was monitored at 220 nm. Forty-five fractions of 2 ml were collected; 1/1000 of each fraction was used in assaying for cell proliferation and cell death in cultured *H. virescens* midgut cells. Active fractions were subsequently injected into a cation-exchange column (TSKgel SP-5PW, 4.6 × 150 mm; Tosoh, Tokyo). A 0.1 *M* gradient of NaCl in 10 m*M* phosphate buffer (pH 6.7) was applied over 90 min at a flow rate of 0.5 ml/min at 40° C. One-milliliter fractions were collected, and 1/100,000–1/500 of each fraction was bioassayed using cultured *H. virescens* midgut cells. Active fractions were then injected on to a reversed-phase (RP) column (Sephasil peptide C18 5u ST 4.6/100; Amersham Pharmacia Biotech, Uppsala, Sweden). A 3–18% gradient of $CH_3CN$ in 0.1% TFA was applied over 40 min at a flow rate of 0.6 ml/min at room temperature. Individual ultraviolet absorption peaks (215 nm) were collected manually; each fraction was bioassayed using cultured *H. virescens* midgut cells. One of the fractions that induced strong inhibition of proliferation was analyzed for amino acid sequence. Amino acid sequence analysis for this peptide was performed by an automated sequencer (PSQ-1; Shimadzu, Tokyo).

Fractions 24 and 25, which stimulated proliferation in the *Mamestra* fat body cell line, separated by the first HPLC step, were applied to a cation-exchange column (TSK gel SP-5PW, 4.6 × 150 mm; Tosoh). A 0.7 *M* gradient of NaCl in 10 m*M* phosphate buffer, pH 6.7, was applied over 90 min at a flow rate of 0.5 ml/min at 40° C. One-milliliter fractions were collected, and 1/50 of each fraction was assayed using the *Mamestra* fat body cell line.

*Bioassays.* Fractions were bioassayed for effects on contractility using a Magnus apparatus with both insect foregut and fish intestine (Sakai et al., in prep.).

Fractions were bioassayed for effects on midgut cells from cultures prepared from *H. virescens* larvae (Loeb and Hakim, 1996). One thousand cells, consisting of stem, columnar, and goblet cells in medium (Loeb and Hakim, 1996), were placed in each well of a 24-well plate (Falcon; Beckton Dickinson, Franklin Lakes, NJ). Each cockroach midgut fraction was added to the wells in duplicate. Control wells received only Ringer's solution. The total number of cells and the number of mature columnar and goblet cells remaining in each well were counted after 6–8 d, and the totals of mature cells were expressed as the percentage of mature cells in control wells. Bioassays of active inhibitory fractions were repeated (N = 4). Active fractions were further bioassayed to determine the dose responses.

The fat body cell line was established from the final larval instars of *M. brassicae* according to the following method formulated by Narita et al. (pers. comm.). Briefly, larvae were surface sterilized with 70% ethanol, allowed to dry under a stream of clean air, and the fat bodies were then removed. Small pieces of tissue were rinsed twice with the culture medium (MGM450), and subsequently transferred to 2 ml MGM-450 medium containing 10% fetal bovine serum (FBS, GIBCO) (Mitsuhashi and Inoue, 1988) in 35-mm plastic tissue-culture Petri dishes (Falcon 3801; Oxnard, CA). The first round of the experiment was conducted using cells of the 17th generation, while the second round of the experiment used cells of the 50th generation. One-thirtieth of the freeze-dried samples of the RP-HPLC fractions of the original midgut homogenate of *P. americana* midgut was added to 500 μl of MGM-450 medium (Mitsuhashi and Inoue, 1988). Cells were cultured in a 96-well microplate at an initial density of $6.8 \times 10^{-4}$ cells in 200 μl of medium at 25° C, in duplicate. Two types of controls were set up, one containing only medium (negative control) and the other containing 10% hemolymph from the fed final-instar larvae of *M. brassicae* which were heated at 60° C for 30 min (positive control). All wells contained 2% of the TCM-1 antibiotic (same as AM-1 antibiotics mixture in Mitsuhashi and Oshiki, 1993). The antibiotic mixture was prepared by mixing and vortexing 1 g dihydrostreptomycin sulfate, 100,000 units penicillin G potassium salt, 1 g kanamycin sulfate (both from Meiji Seika, Tokyo, Japan), 0.1 g novobiocin (Difco Laboratories, Detroit, MI), and 50 ml distilled water that contained 10% FBS.

## Results

*Effects on* H. virescens *midgut cells.* Figure 1*A* shows the first RP-HPLC profile obtained from the extract of *P. americana* midguts. Some fractions showed activities modifying foregut contraction (Fig. 1*B*). These fractions were tested on *Heliothis* midgut cultures for their cell proliferation and depletion activities (Fig. 1*C*). Fractions 12 and 13 induced 5 ± 2 and 18 ± 8% (standard error of the mean [SEM]) proliferation, respectively. Fractions 16 and 17 caused 50 ± 2.9 and 61.8 ± 1.3% decrease, respectively, in the number of mature columnar and goblet cells in *H. virescens* midgut cultures, when examined 5 and 8 d after the addition of the peptide fraction (Fig. 1*C*). The inhibitory material probably induced cell death in the mature cells of each exposed population. The combined fractions 16–17 were further separated by cation exchange (Fig. 2). Bioassays of fractions 20, 31, and 42 indicated that 70 ± 2% of the mature columnar and goblet cells were depleted. Figure 3 shows the dose responses to the active fractions 20 (*circles*), 31 (*squares*), and 42 (*triangles*) in the cation-exchange chromatography shown in Fig. 2. U-shaped dose dependency is typical for peptide–cell interactions (Wagner et al., 1997). These fractions were further separated individually in the third RP-HPLC. Fractions 20 and 21, derived from fraction 20 of the cation exchange, caused 80 ± 0% depletion; fractions 6 and 7 from fraction 31 caused 76 ± 3% depletion (data not shown). Fractions 16 and 17 from fraction 31 of the cation exchange caused 80 and 78 ± 2% (SEM) depletion of columnar and goblet cells, respectively (Fig. 4). Fraction 17 was sequenced by Edman degradation since it showed a clean single peak unlike fraction 16. Amino acid sequence was partially determined as Ala-Val-()-Lys-Ala-()-()-()-()-()-Val-(). Further studies on this peptide are in progress.

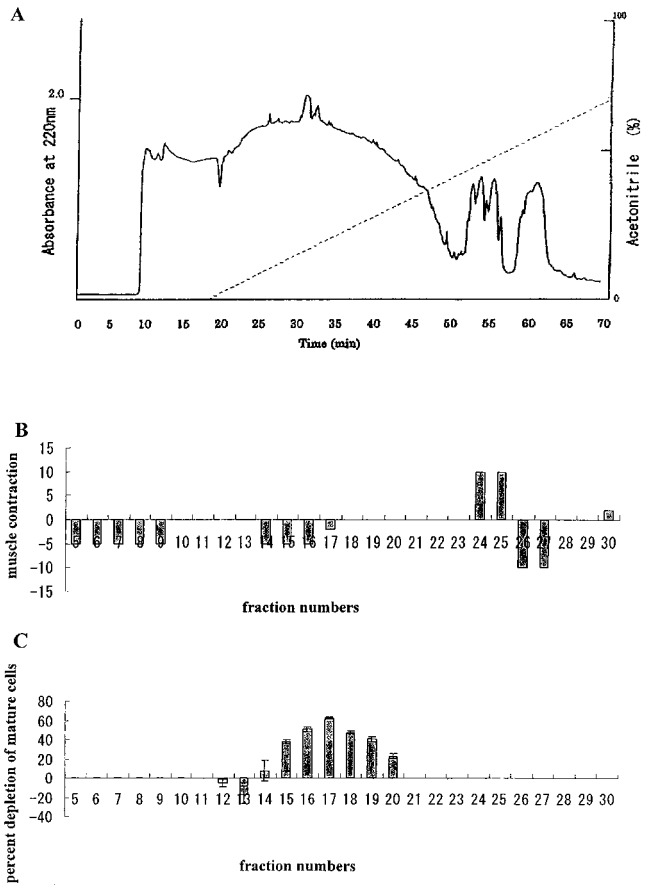


FIG. 1. An RP chromatography of *P. americana* midguts (*A*) and the effect on *Teleogryllus occipitalis* foregut contraction (*B*), where the effects are expressed as relative intensity to untreated control, 1.0–10% increase from the control, and on the numbers of cultured midgut cells from *H. virescens* larvae (*C*). Cells were counted 5 d after the addition of 1/1000 of each peptide fraction.

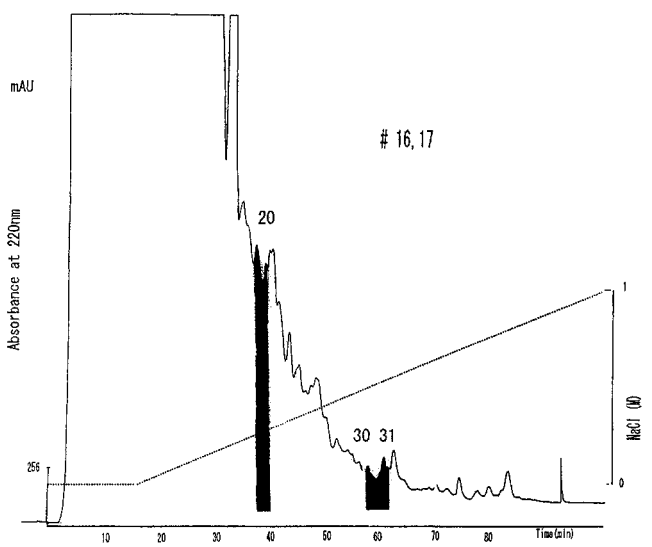


FIG. 2. Cation-exchange chromatography of fractions 16 and 17 in Fig. 1. *Shaded peaks* show cultured *H. virescens* midgut depletion.

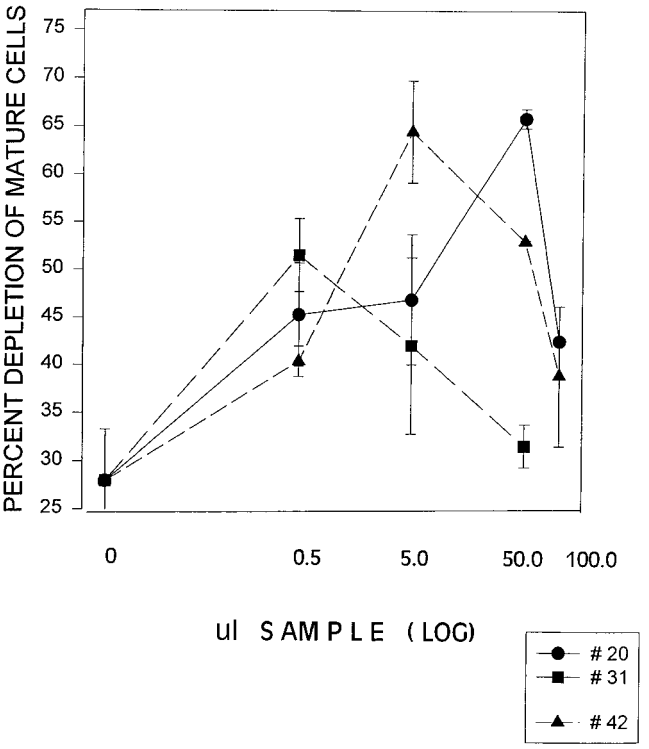


FIG. 3. Dose responses of *H. virescens* midgut cells to active fractions 20 (*circles*), 31 (*squares*), and 42 (*triangles*) derived from the chromatography seen in Fig. 2. N = 3 (± SEM) for each *point*.

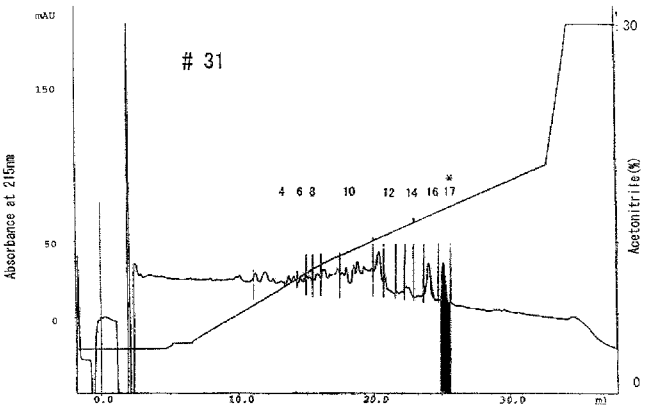


FIG. 4. The second RP-chromatography profile of active fraction 31 shown in Fig. 2*A*. The *shaded peak*, fraction 17, was sequenced.

*Effect on fat body cell line.* Fractions 24 and 25, separated by the first step RP-HPLC, stimulated cell proliferation in the fat body cell line (data not shown). These fractions also strongly inhibited the spontaneous contraction of the foregut of *Teleogryllus occipitalis* (Fig. 1*B*). They were combined and further separated by cation-exchange HPLC (Fig. 5). Two of the fractions (16 and 26) showed cell-proliferative activity (Fig. 5). The rates of proliferation were 14 and 18 times that of an untreated control, respectively. Myoinhibitory activities were found in fractions 18–19 and 24–25.

Active fractions 16 and 26 were further separated by the second RP-HPLC, and tested for proliferative activity. Further separation and analysis of these peptides is currently in progress.

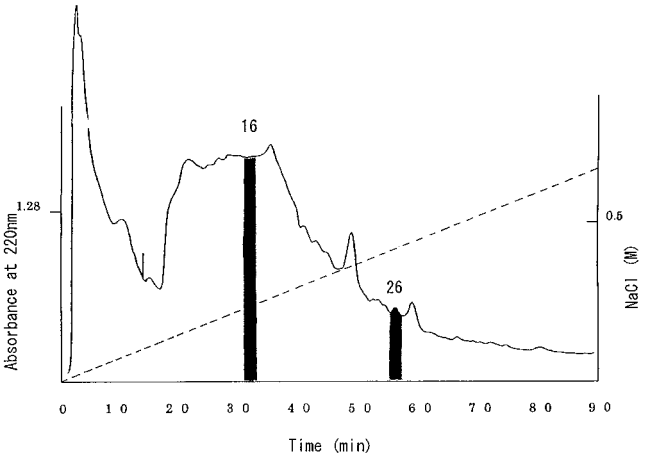


FIG. 5. Cation-exchange chromatography of active fractions 24 and 25 shown in Fig. 1. *Shaded fractions* 16 and 26 in this figure indicate fat body cell proliferation activity. Myoinhibitory activities were found in flanking fractions 18–19 and 24–25.

## DISCUSSION

Our results showed that the cockroach midgut contained several peptide growth factors having both stimulatory and inhibitory activities on cell proliferation, differentiation, and death. Some peptides caused proliferation of cultured lepidopteran midgut and fat body cells. Others induced depletion of mature midgut cells, suggesting that they stimulated apoptosis in these cell types, and/or that they suppressed the rate of stem cell proliferation and differentiation that effects replacement of lost mature cells. Replacement normally occurs when mature midgut cells are killed by toxins (Loeb et al., 2000). Cockroach factors seemed to be neither species- nor tissue-specific since they affected the lepidopteran midgut as well as the fat body cells. They may have multiple functions since the peptides in fractions that inhibited muscular activity in the cricket foregut assay also induced depletion of mature *H. virescens* cells.

In our laboratory, we have also identified peptides identical to crustacean cardioactive peptide (CCAP) and two allatostatins (ASHs) in cockroach midgut homogenates using the foregut contraction assay (Sakai et al., in prep.). These peptides may have multiple functions because CCAP from the midgut may regulate the heart and ASHs may control the corpora allata. The midgut may be a rich source of bioactive molecules as well as a center of coordination for various physiological functions occurring in various organs via intricate intercommunication between endocrine factors, including peptidic and nonpeptidic neuromediators, and interactions with neural elements. Communication between endogenous midgut factors and fat body, and gonads deserves future investigations.

## ACKNOWLEDGMENTS

We thank Prof. Y. Muneoka, Mr. E. Clark, Dr. N. Ichihara, and Mr. S. Goto for technical support and valuable comments. A grant support by 'Research for Future' Program from JSPS (99L01205) is appreciated.

## REFERENCES

Day, M. F.; Powning, R. F. A study of the processes of digestion in certain insects. Aust. J. Biol. Sci. 2:175–221; 1949.

Dow, J. A.; Harvey, W. R. Role of midgut electric K+ pump potential difference in regulating lumen K+ and pH in larval Lepidoptera. J. Exp. Biol. 140:455–463; 1988.

Endo, Y.; Iwanaga, T.; Fujita, T., et al. Localization of pancreatic polypeptide (PP)-like immunoreactivity in the central and visceral nervous systems of the cockroach *Periplaneta*. Cell Tissue Res. 227:1–9; 1982.

Ferkovich, S. M.; Oberlander, H. Growth factors in invertebrate in vitro culture. In Vitro Cell. Dev. Biol. 27A:483–486; 1991.

Homma, K.; Matsushita, T.; Natori, S. Purification, characterization, and cDNA cloning of a novel growth factor from conditioned medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga perigrina* (flesh fly). J. Biol. Chem. 271:13,770–13,775; 1996.

Kawamura, K.; Shibata, T.; Saget, O., et al. A new family of growth factors produced by the fat body and activate on *Drosophila* imaginal discs. Development 126:211–219; 1999.

Loeb, M. J.; Hakim, R. S. Insect midgut epithelium in vitro: an insect stem cell system. J. Insect Physiol. 42:1103–1111; 1996.

Loeb, M. J.; Hakim, R. S. Cultured midgut cells of *Heliothis virescens* (Lepidoptera): fibronectin and integrin β 1 immunoreactivity during differentiation in vitro. Invertebr. Reprod. Dev. 35:95–102; 1999.

Loeb, M. J.; Hakim, R. S.; Martin, P., et al. Apoptosis in cultured cells from *Heliothis virescens* larvae exposed to various conditions. Arch. Insect Biochem. Physiol. 45:12–23; 2000.

Loeb, M. J.; Jaffe, H.; Gelman, D. B., et al. Two polypeptide factors that promote differentiation of insect midgut stem cells in vitro. Arch. Insect Biochem. Physiol. 40:129–140; 1999.

Mitsuhashi, J.; Inoue, H. Obtainment of a continuous cell line from the larval fat bodies of the mulberry tiger moth, *Spilosoma imparilis* (Lepidoptera: Arctiidae). Appl. Entomol. Zool. 23:488–490; 1988.

Mitsuhashi, J.; Oshiki, T; Preliminary attempts to rear an endoparasitic fly, Exorista sorbilans (Diptera, Tachinidae) in vitro. Jpn J. Entomol. 61:459–464.

Neal, J. J.; Reuveni, M. Separation of cytochrome P-450 containing vesicles from the midgut microsomal fraction of *Manduca sexta*. Comp. Biochem. Physiol. 102C:77–82; 1992.

Nishiitsutsuji-Uwo, J.; Endo, Y.; Takeda, M. Insect brain–midgut endocrine system: with special reference to paraneurons. In: Kobayashi, H., et al., ed. Neurosecretion and the biology of neuropeptides. New York: Springer; 1985:410–417.

Nishiitsutsuji-Uwo, J.; Endo, Y.; Takeda, M. Brain–gut peptides in the cockroach with special reference to midgut. In: Stefano, J., ed. CRC handbook of comparative opioid and related neuropeptide mechanisms. Vol. 2. New York: CRC Press; 1986:81–91.

Palli, S. R.; Locke, M. The synthesis of hemolymph proteins by the larval midgut of an insect *Calpodes ethlius* (Lepidoptera: Hesperiidae). Insect Biochem. 17:561–572; 1987.

Sehnal, F.; Zitnan, D. Endocrines of insect midgut. In: Progress in comparative endocrinology. New York: Wiley-Liss; 1990:510–515.

Strand, M. R.; Hayakawa, Y.; Clark, K. D. Plasmatocyte spreading peptide (PSP1) and growth blocking peptide (GBP) are multifunctional homologs. J. Insect Physiol. 46:817–824; 2000.

Takeda, M.; Yoshida, Y.; Endo, Y. Serotonin is "the midgut regeneration stimulating factor (MRSF)" in *Periplaneta*. Physiol. Zool. 68:111; 1995.

Taylor, C. W. Calcium absorption by the midgut of the Blowfly, *Calliphora vicina*. J. Exp. Biol. 114:551–561; 1985.

Teo, L. H.; Woodring, J. P. The invertase of the house cricket *Acheta domesticus* L. (Orthoptera: Gryllidae). Comp. Biochem. Physiol. 93B:643–646; 1989.

Valaitis, A. P. Gypsy moth midgut proteinases: purification and characterization of luminal trypsin, elastase, and the brush border membrane leucine aminopeptidase. Insect Biochem. Mol. Biol. 25:139–149; 1995.

Valaitis, A. P.; Boweres, D. F. Purification and properties of the soluble midgut trehalase from the gypsy moth, *Lymantria dispar*. Insect Biochem. Mol. Biol. 23:599–606; 1993.

Veenstra, J. A.; Lau, G. W.; Agricola, H.-J., et al. Immunohistological localization of regulatory peptides in the midgut of the female mosquito *Aedes aegypti*. Histochem. Cell Biol. 104:337–347; 1995.

Verhaert, P.; Huybrechts, R.; Schols, D., et al. Characterization of vertebrate peptide hormone-like materials in the American cockroach: different methods employed to investigate these substances and their results. In: Borkovec, D. B.; Gelman, A. B., ed. Insect neurochemistry and neurobiology. New Jersey: Humana Press; 1986:229–233.

Wagner, R. M.; Loeb, M. J.; Kochansky, J. P., et al. Identification and characterization of an ecdysiotropic peptide from brain extracts of the Gypsy moth, *Lymantria dispar*. Arch. Insect Biochem. Physiol. 34:175–189; 1997.

Weirich, G. Ecdysone 20-hydroxylation in *Manduca sexta* (Lepidoptera: Sphingidae) midgut: development-related changes of mitochondrial and microsomal ecdysone 20-monooxygenase activities in the fifth larval instar. Eur. J. Entomol. 94:57–65; 1997.

Weirich, G.; Thompson, M. J.; Svoboda, J. A. In vitro ecdysteroid conjugation by enzymes of *Manduca sexta* midgut cytosol. Arch. Insect Biochem. Physiol. 3:109–126; 1986.